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Short-Term Metallothionein and Copper Changes in Blue Crabs at Ecdysis

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Abstract. We have previously demonstrated that the small metal-binding protein, metallothionein (MT), plays an important role in the metabolism of Cu and Zn during the molt cycle of the blue crab, *Callinectes sapidus*. To further delineate the role of MT in the regulation of both metals, the distribution of copper and zinc was examined immediately after ecdysis in the blue crab. Hemolymph, digestive gland, and stomach were analyzed, by atomic absorption spectrophotometry (AAS), for total metal concentration in crabs at different molt stages, from premolt (D_3) through paper shell (B_2), and including intermolt (C_4). Cytosolic extracts were prepared from digestive glands of individual crabs and analyzed, by gel filtration chromatography and AAS, for MT, copper, and zinc. The short-term changes in metal concentrations in the tissues, and those in MT and metals in the cytosol were dramatic. Transient changes in the metals bound to MT correlated well with the loss of copper from the hemolymph and the digestive gland. The observed changes occurred over a period of 90 min after ecdysis. The data suggest that copper is stripped from hemocyanin in the digestive gland after ecdysis, displacing zinc from MT in the cytosolic pool. We hypothesize that the copper/zinc-MT complex may then be sequestered in lysosomes and eliminated into the gut and out in the feces. A discriptive flow model showing the involvement of MT in copper and zinc partitioning after ecdysis in the blue crab has been constructed.

Introduction

Recent investigations have demonstrated that molting in the blue crab, *Callinectes sapidus*, profoundly affects the tissue and cytosolic concentrations and partitioning of copper and zinc (Engel, 1987; Engel and Brouwer, 1987). At molt the concentration of circulating hemocyanin, the copper-containing respiratory protein of crustaceans, decreases dramatically. In the blue crab this decrease is about 60% (Mangum *et al.*, 1985; Engel, 1987). Because hemocyanin is a large copper-containing protein that occurs in high concentrations (~50 mg/ml corresponding to 0.67 m*M* protein and 1.33 m*M* of copper), its degradation releases significant amounts of copper into the cytosolic metal pools. The rapidity of the events, and the reactivity of the copper, dictate that some mechanism must be present to detoxify the copper and to assist in the excretion or storage of the metal. In our earlier investigations, we have attempted to account for the fate of the released copper, but have been unable to find any pool of the metal stored in the tissues of animals in the papershell or early hard crab stages. These results suggested that the excess copper may be excreted.

The low molecular weight metal-binding protein, metallothionein (MT), also changes in concentration and in metal composition in relation to the molt cycle (Engel, 1987; Engel and Brouwer, 1987). The changes that have been observed in MT correlate with the metabolic requirements for copper and the synthesis and turnover of hemocyanin (Engel and Brouwer, 1987; Brouwer *et al.*, 1989). Cu-MT from marine crustaceans can be separated into three different forms (Brouwer *et al.*, 1986). The Cu-MT(1) and Cu-MT(2) isoforms cannot reactivate apohemocyanin *in vitro* (Brouwer *et al.*, 1989). However, Cu-MT(3), which differs in amino acid composition from MT(1) and MT(2), can serve as a copper donor for apohemocyanin, and can reconstitute its oxygen-binding function (Brouwer *et al.*, 1989).

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We suspected that, during the breakdown of hemocyanin, the liberated copper is bound to MT and excreted from the crab. To test this hypothesis, we examined blue crabs just prior to, during, and immediately after ecdysis to determine how the copper is excreted, and to elucidate the mechanism and time course of the process.

Materials and Methods

All of the premolt and postmolt crabs used in these experiments were obtained from commercial blue crab shedding operations at Beaufort, North Carolina. The premolt crabs were selected at the site and transported to the laboratory for sampling of tissues. Tissue samples from postmolt animals were collected on site at different times after ecdysis. Hemolymph samples were placed on ice, and the digestive gland samples were frozen on dry ice. The molt stages that were sampled in this investigation were: premolt, D_3 and D_4; soft crab, A_1 and A_2; and papershell B_1 and B_2. The timed tissue samples were taken from A_1 stage crabs at 0, 15, 45, 60, and 90 min after ecdysis.

The concentrations of copper and zinc were determined in samples of digestive gland, stomach, and hemolymph from individual blue crabs. The hemolymph samples were collected by severing an appendage between two joints and collecting the hemolymph in polyethylene vials. Hemolymph was analyzed for hemocyanin, copper, and zinc concentrations (Engel and Brouwer, 1987). Digestive glands and stomachs were collected for total metal analysis. Portions of the digestive glands were stored at −70°C for the determination of the cytosolic distribution of metals and metallothioneins.

Tissue samples used for metal analysis were dried at 100°C for 24 to 48 h and wet ashed with concentrated HNO_3 at 90°C. The residue was dissolved in 0.25 *N* HCl, and the concentrations of copper and zinc were measured by flame atomic absorption spectrophotometry. Preparative and measurement techniques were calibrated against the National Bureau of Standards, Oyster Reference Material #1566.

The cytosolic distribution of copper, zinc, and MT was determined by gel filtration chromatography with Sephadex G-75 (Engel, 1987). In these investigations, a computer program was developed that allows for the averaging of elution profiles so that there is less subjectivity in the evaluation of results. The program requires the use of uniform methodologies, and provides metal concentrations in each fraction in terms of micromoles of metal per kilogram wet weight of tissue.

Data on molt-induced changes in tissue metal concentrations were tested for significance ($P < 0.05$) by analysis of variance and Tukey's studentized range test. The cytosolic distributions of copper and zinc were analyzed with the assistance of a computer program developed in our Laboratory that gave average elution profiles.

Results

The concentrations of hemocyanin, copper, and zinc in the hemolymph, and of copper and zinc in the digestive gland, varied with molt stage (Figs. 1, 2). Significant changes occurred throughout the molt cycle, in the level of hemocyanin, and in the concentrations of copper and zinc in the hemolymph and digestive gland (ANOVA, $P < 0.05$). In the hemolymph, concentrations of hemocyanin, copper, and zinc decreased significantly ($P < 0.05$) at ecdysis, between D_4 and A_1, and remained at reduced levels throughout the papershell stage. There was some indication that the hemocyanin concentration was increasing at the end of the papershell stage (Fig. 1). In the digestive gland, a transient, significant ($P < 0.05$) increase in copper concentration occurred in stage A_1 relative to stages D_4 and A_2 (Fig. 2). This increase in copper concentration in the digestive gland coincides with the decreases in the concentrations of both hemocyanin and copper in the hemolymph. The subsequent decrease in copper concentration in A_2 suggests that the copper is being lost from the tissue.

The transient increase in copper during A_1 is not seen with zinc (Figs. 1, 2). When the changes in copper and zinc in the digestive gland are compared on a molar basis, an increase of about 0.3 m*M*/kg in copper is revealed between D_4 and A_1, and a concomitant 0.3 m*M*/kg decrease occurs in zinc (Fig. 2). However, this copper/zinc relation does not hold as the crabs go from A_1 to A_2. The data suggest that, during those stages of the molt cycle, both copper and zinc are lost from the digestive gland.

To determine the possible route of metal loss by the crabs during the period following ecdysis, stomachs of hard (C_4), soft (A_1–A_2), and papershell (B_1–B_2) crabs were examined for concentrations of copper and zinc (Fig. 3). The data from these measurements show that the stomachs of soft crabs have significantly higher concentrations of copper and zinc ($P < 0.05$) than those of either hard crabs or papershell crabs, which were not significantly different. Such information supports the idea that the pathway for the loss of copper and zinc following ecdysis leads from the digestive gland to the gut, and that the metals are excreted in the feces (*e.g.*, digestive gland → stomach → gut → feces).

Because the changes in copper and zinc concentrations in the digestive gland apparently occur quite rapidly, short-term measurements were made of the cytosolic partitioning of copper and zinc (*i.e.*, the portion of metals bound to MT). Although the total amount of copper changed, the partitioning of copper was similar at each sampling time. The maximum amount of copper was bound to MT

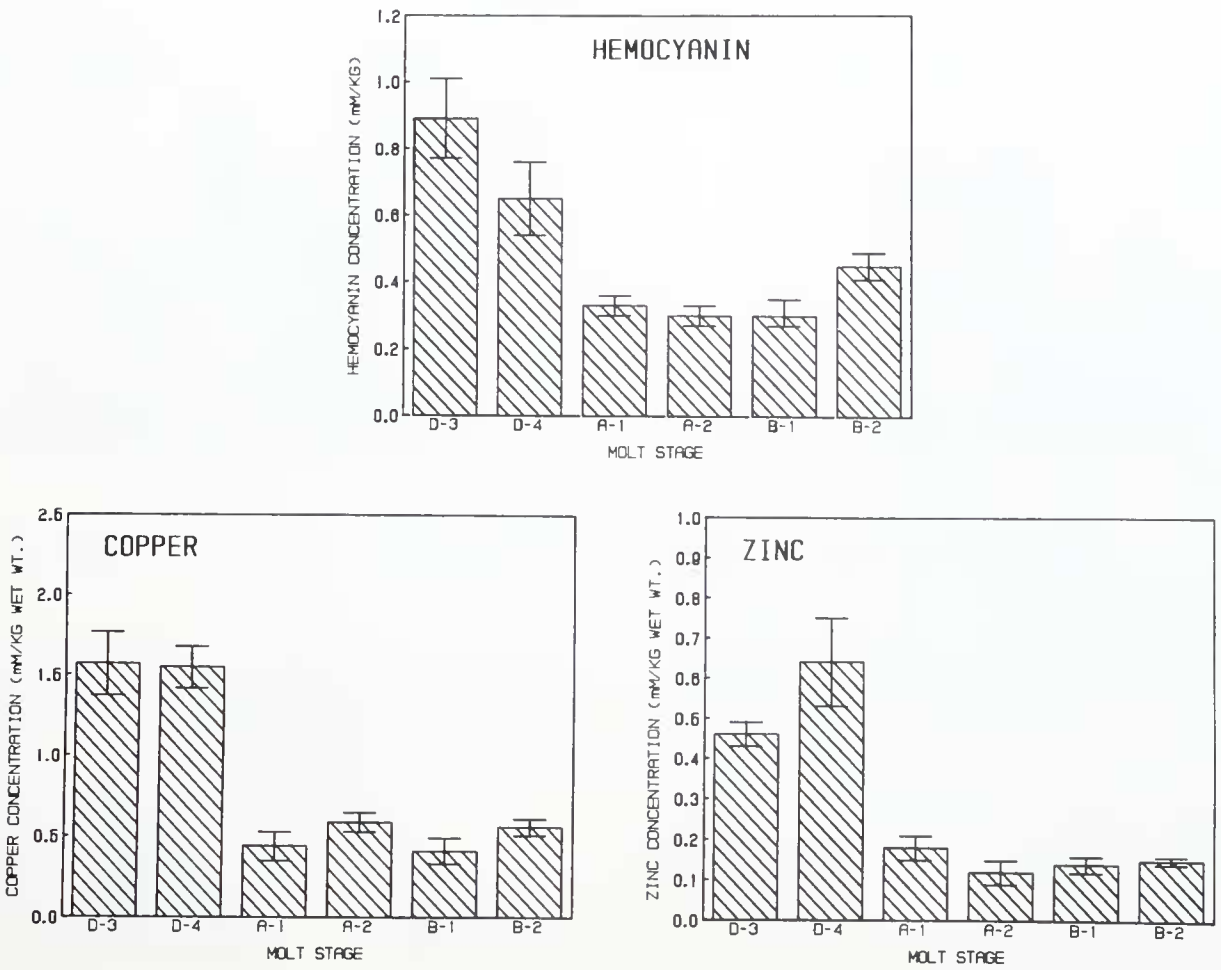

Figure 1. Histograms showing the average concentrations of hemocyanin, copper and zinc in the hemolymph at selected stages of the molt cycle. All concentrations of hemocyanin, copper and zinc are reported in millimoles/kilogram wet weight of sample. Each bar represents the mean of six crabs and the vertical lines above and below the mean describe one standard error.

60 min after ecdysis (Fig. 4). While the amount of Cu-MT increased in the cytosol from 15 through 60 min after ecdysis, the amounts of Zn-MT decreased over the same period. In the period between 45 and 60 min after ecdysis, there was about a 10 micromolar increase in copper bound to MT and a similar decrease in the amount of bound zinc. This observation suggests that zinc was displaced from the MT by copper released during the rapid degradation of hemocyanin following ecdysis. Cytosolic copper concentrations were initially low, but had increased five fold by 60 min after ecdysis (Fig. 5), which correlates well with the observed increase in copper bound to MT (Fig. 4). There was very little change in cytosolic zinc concentration for the first 60 min after ecdysis, but it did decrease between 60 and 90 min. These decreases in both Cu and Zn-MT and in total cytosolic copper between 60 and 90 min suggests again that metal was lost from the digestive gland at this time.

Discussion

From the data collected in our current and earlier experiments (Engel, 1987; Engel and Brouwer, 1987) on the mechanisms of copper and zinc metabolism during the molt cycle of the blue crab, we have constructed a diagram showing the relationships between the breakdown of hemocyanin and the changes in the concentrations of Cu-MT and Zn-MT (Fig. 6). Three significant changes occur in the metals bound to MT during the molt cycle. The first is at the beginning of premolt when the metals bound to MT change from predominantly copper to zinc. The second occurs within 90 min after ecdysis when there is a transient pulse of copper bound to the predominantly zinc-MT. The third change occurs during stages B_1 and B_2 when the MT once again becomes primarily a copper protein, and this change is correlated with synthesis of hemocyanin (Engel and Brouwer, 1987).

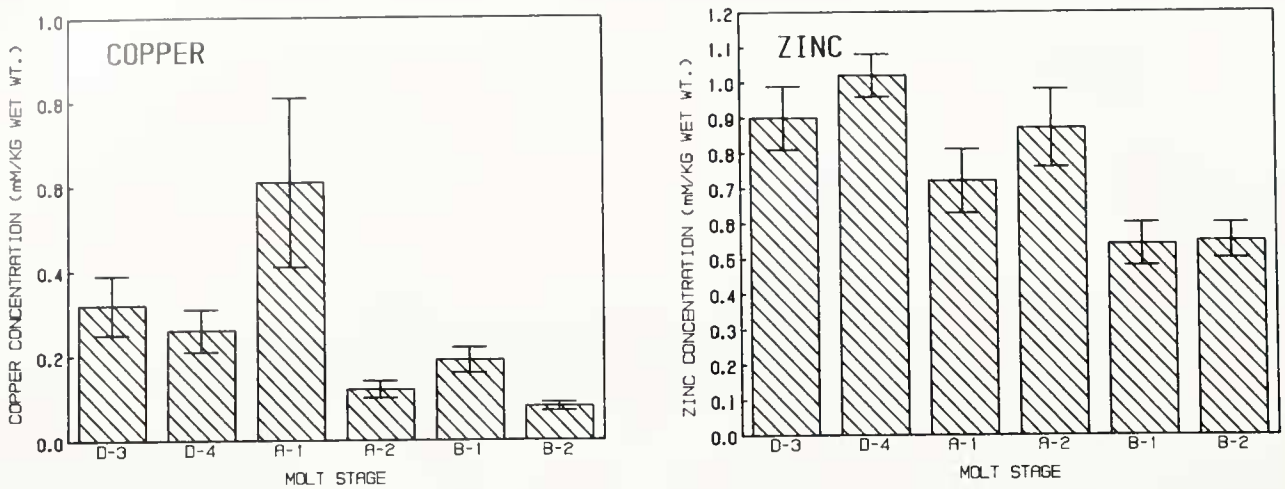

Figure 2. Histograms showing the copper and zinc concentrations in digestive glands of blue crabs at selected stages of the molt cycle. All concentrations and error designations are the same as described in Figure 1.

At the end of the intermolt stage, C_4, and the beginning of premolt, D_1, the metal bound to MT changes from predominantly copper to zinc. The trigger for this change has yet to be demonstrated. We hypothesize, however, that the reduction in the concentrations of Cu-MT is correlated with reduced hemocyanin synthesis and an increased rate of Zn-carbonic anhydrase synthesis in preparation for molting. These types of changes, which preceed molting, could be initiated by increases in the concentration of the molting hormone, ecdysteroid (Soumoff and Skinner, 1983). While the magnitude of the changes in the metals bound to MT are large, from 90% copper to 90% zinc (Engel, 1987), no information is available on either the timing or rate of the change.

The transient pulse of copper bound to MT in the digestive gland cytosol immediately after ecdysis (*i.e.*, within 90 min) is undoubtedly correlated with the catabolism of hemocyanin. Because there is roughly a 60% decrease in hemocyanin concentration in the hemolymph shortly after molt (Fig. 1) (Mangum *et al.*, 1985; Engel, 1987), a large quantity of copper should be released into the cytosol of the digestive gland in a relatively short time. The observed pulse of Cu-MT, therefore, represents the detoxification of the liberated copper by an *in situ* processes in the digestive gland cytosol. To more fully describe our hypothesis, a flow diagram has been developed that shows the interaction between the released copper and the cytosolic pool of Zn-MT present at ecdysis (Fig. 7). The mechanism of copper detoxification may be a straightforward substitution process involving the pool of Zn-MT already present in the digestive gland. This large concentration of Zn-MT in the cytosol acts as a sink for the copper that is released during the degradation of hemocyanin. Because copper has a higher binding affinity for MT than does zinc, it simply displaces the zinc already bound to MT. This process would account for the rapid kinetics, because *de novo* synthesis of MT is unlikely to occur rapidly (Hildebrand and Enger, 1980). This substitution process will not result in an all-copper protein. After the substitution, a significant portion of the Cu/Zn-MT complex is excreted via lysosomes into the digestive tract and out in the feces, and the remainder may serve as the initial copper donor for renewed hemocyanin synthesis. The excess zinc not bound to MT can either be excreted

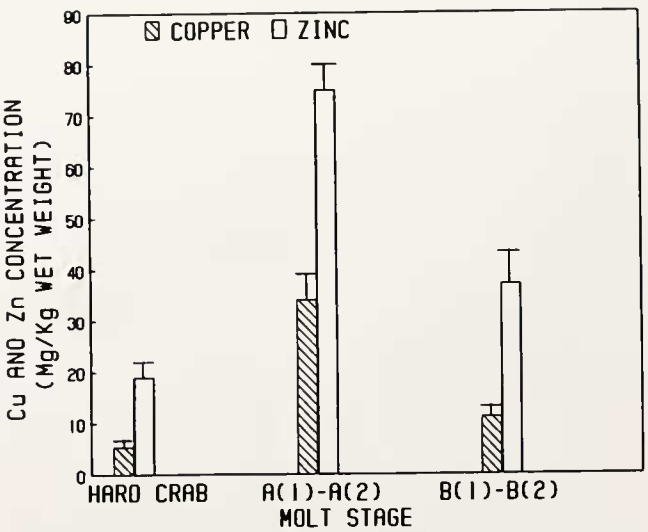

Figure 3. Histograms showing the copper and zinc concentrations in the stomachs of hard crabs [n = 8], soft crabs (A_1 and A_2) [n = 5], and papershell crabs (B_1 and B_2) [n = 6]. Concentrations are given as milligrams of metal per kilogram of tissue plus and minus one standard error of the mean.

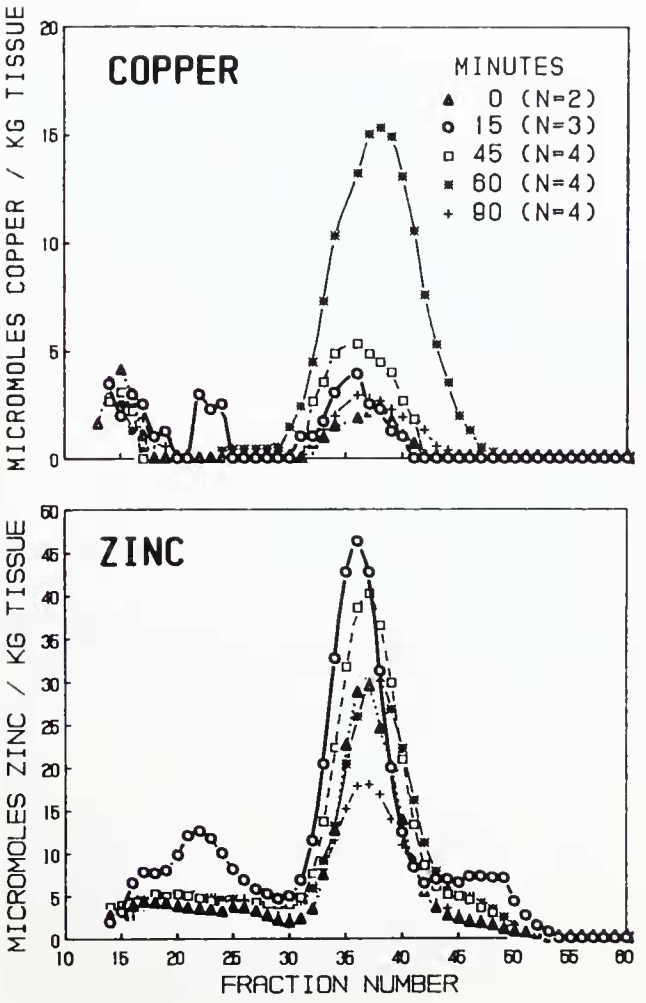

Figure 4. Average elution profiles of protein bound copper and zinc in digestive gland cytosols at five specific times after ecdysis (0, 15, 45, 60, and 90 min). Each elution profile is a computer generated average of from 2 to 4 individual crabs. The concentrations of copper and zinc are normalized to the wet weight of the tissue used and to the amount of cytosol applied to the column.

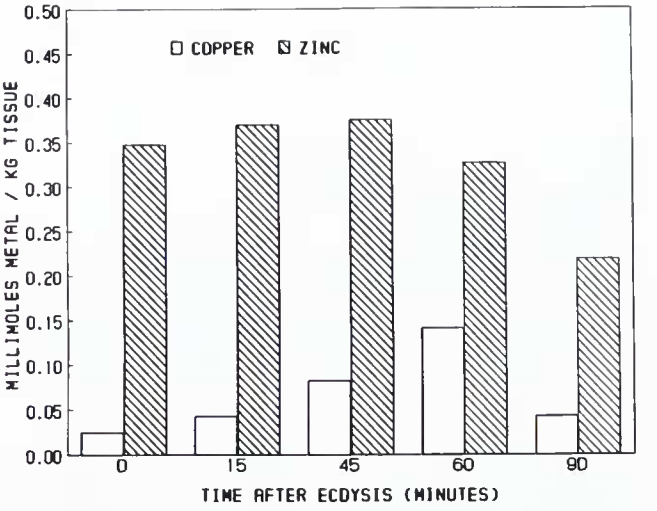

Figure 5. Histograms showing the average cytosolic concentrations of copper and zinc (*i.e.*, millimoles/kg) in digestive glands at five times after ecdysis (0, 15, 45, 60, and 90 min). These metal concentrations are calculated from the cytosolic samples applied to the Sephadex G-75 column.

in lysosomes via the gut, or via the green gland in the urine.

Our functional model of copper detoxification agrees with the observations made by Al-Mohanna and Nott (1989) on the shrimp *Penaeus semisulcatus*. In their electron microscopic examination of the shrimp hepatopancreas during the molt cycle, they demonstrated the presence of copper and sulfur containing granules in the R cells of the hepatopancreas using EDAX energy dispersive microanalysis. These granules are released to the lumen of the hepatopancreas through cellular degeneration and sloughing. The occurrence of these copper containing granules may be associated with the synthesis and turnover of hemocyanin.

The conversion of the MT back to a copper protein occurs later in the postmolt period, B_1 and B_2, and precedes the resynthesis of hemocyanin (Engel, 1987; Engel and Brouwer, 1987), strongly suggesting that Cu-MT may act, directly or indirectly, as the source of copper for hemocyanin synthesis (Fig. 7). Brouwer and coworkers (1986, 1989) have demonstrated that Class-I Cu-MTs [*i.e.*, related in primary structure to equine renal MT (Fowler *et al.*, 1987)] isolated from the hepatopancreas of the American lobster cannot transfer their copper to hemocyanin. However, a third copper-protein, which has a lower molecular weight than the Class-I MTs, contains less cysteine, and is much more acidic, has been isolated from the lobster hepatopancreas. This copper-protein,

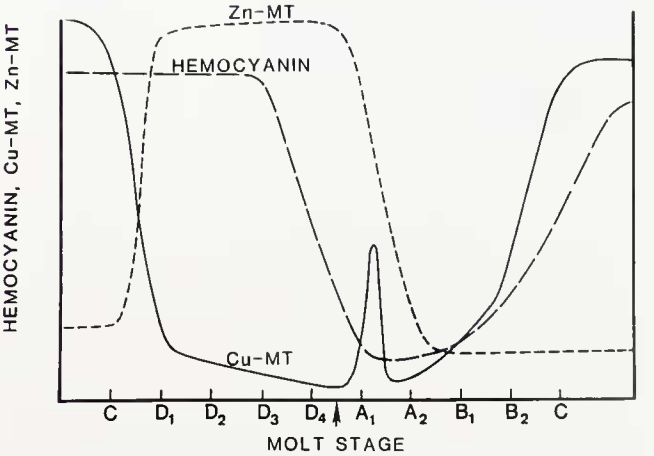

Figure 6. A descriptive diagram showing the relationships between hemocyanin in the hemolymph and copper and zinc MT during the molt cycle of the blue crab. The arrow denotes the time of ecdysis.

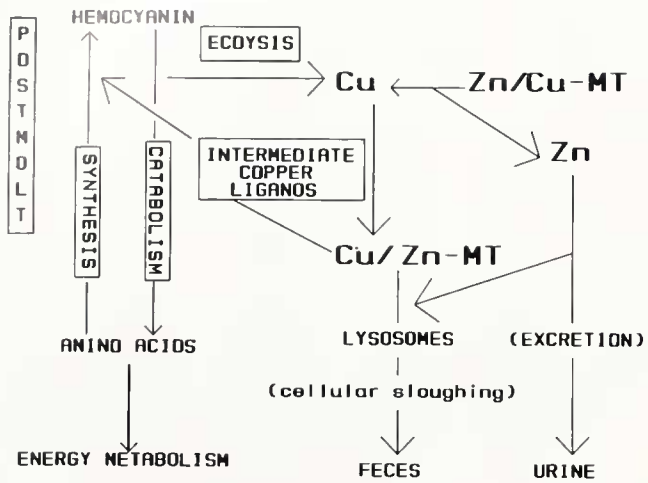

Figure 7. A flow diagram of the synthetic and catabolic pathways for hemocyanin and the interactions between copper, zinc, and copper/zinc-MT in the digestive gland of the blue crab immediately after ecdysis and during the later postmolt recovery period. The diagram includes the pathways for detoxification of copper released from hemocyanin, excretion of copper and zinc (Engel, 1987; Engel and Brouwer, 1987; and Al-Mohanna and Nott, 1989), and the presence of a lower molecular weight compound that is active in the transfer of copper to the apoprotein during hemocyanin synthesis (Brouwer, unpub. data).

tentatively classified as a Class II-MT, has been found effective in restoring the oxygen binding capacity of apohemocyanin (Brouwer *et al.*, 1989). Whether copper exchange occurs between the Class I and II-MTs remains to be demonstrated.

This investigation gives further support to the hypothesis that the function of metallothionein in normal organisms is in the regulation of nutritional metals. Through the use of the normal crustacean growth process of molting, we have been able to identify some of the functional mechanisms of cytosolic metal regulation involving MT. These data also serve to point out that if a protein such as MT is to be used as an indicator of animal health, the processes that control its abundance and turnover must be demonstrated.

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Literature Cited

Al-Mohanna, S. Y., and J. A. Nott. 1989. The accumulation of metals in the hepatopancreas of the shrimp *Penaeus semisulcatus* de Hann (Crustacea; Decapoda) during the molt cycle. Pp. 195–207 in *Proceedings of the First Arabian Conference on Environment and Pollution.* Allen Press, Oxford.

Brouwer, M., D. R. Winge, and W. R. Gray. 1989. Structural and functional diversity of copper-metallothionein from the American lobster, *Homarus americanus. J. Inorg. Biochem.* **35:** 289–303.

Brouwer, M., P. Whaling, and D. W. Engel. 1986. Copper-metallothioneins in the American lobster, *Homarus americanus:* potential role as Cu(I) donors to apohemocyanin. *Environ. Health Perspect.* **65:** 93–100.

Engel, D. W. 1987. Metal regulation and molting in the blue crab, *Callinectes sapidus:* copper, zinc, and metallothionein. *Biol. Bull.* **172:** 69–82.

Engel, D. W., and M. Brouwer. 1987. Metal regulation and molting in the blue crab, *Callinectes sapidus:* metallothionein function in metal metabolism. *Biol. Bull.* **173:** 237–249.

Fowler, B. A., C. E. Hildebrand, Y. Kojima, and M. Webb. 1987. Nomenclature of metallothionein. *Experientia Suppl.* **52:** 19–22.

Hildebrand, C. E., and M. D. Enger. 1980. Regulation of Cd^{2+}/Zn^{2+}-stimulated metallothionein synthesis during induction, deinduction, and superinduction. *Biochemistry* **19:** 5850–5857.

Mangum, C. P., B. R. McMahon, P. L. DeFur, and M. G. Wheatly. 1985. Gas exchange, acid-base balance, and the oxygen supply to the tissues during a molt of the blue crab, *Callinectes sapidus. J. Crust. Biol.* **5:** 188–206.

Soumoff, C., and D. M. Skinner. 1983. Ecdysteroid titers during the molt cycle of the blue crab resembled those of other crustacea. *Biol. Bull.* **165:** 321–329.